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Degradation of Chlorinated herbicides by *Klebsiella pneumoniae* from Rhizosphere of Rice

Kim, Jin-Woong, Sung-Ho Bang, Sung-Sub Park, Sang-Kyun Koh and Yung-Nok Lee

Department of Biology, Korea University

벼의 근권으로부터 분리한 *Klebsiella pneumoniae*에 의한 제초제의 분리

김진웅 · 방성호 · 박성섭 · 고상균 · 이영록

고려대학교 생물학과

Abstract: It was observed that the strains of *Klebsiella pneumoniae* isolated from rhizosphere of rice, capable of utilizing chlorinated herbicides, such as 2, 4-dichlorophenoxyacetate, 2-methyl-4-chlorophenoxyacetate and 3-chlorobenzoate, as sole source of carbon and energy and confirmed that their degrading ability of the herbicides was due to plasmid genes. Characteristics of selected strains such as nitrogenase activity, resistances to antibiotics and heavy metal ion were measured.

Key words: *Klebsiella pneumoniae*, rhizoshere.

N_2 fixation in the *rhizosphere* of grasses and non-leguminous crops was indicated some time ago(Parker, 1957). The first undisputable report on this process was that of Hassouna and Wareing (1964) who found active N_2 fixation in the rhizosphere of *Ammophila arenaria*, Which they attributed to *Azotobacter* associated with roots. The report of high levels of nitrogen-fixing activity associated with isolated roots of maize was by von Bulow and Dobereiner (1975). Hirota *et al.* (1978) found that some bacteria in the rhizophere of rice fix nitrogen, and some of the bacteria were isolated and identified as *Klebsiella oxytoca* by Komagata *et al.* (personal communication).

Chlorinated phenoxyherbicides such as 2, 4-dichlorophenoxyacetic acid(2, 4-D) and 2, 4, 5-trichlorophenoxyacetic acid(2, 4, 5-T) have been used extensively in agriculture for over 40 years. Recently, several groups of workers have reported the isolation of bacteria belonging to different genera that are capable degrading these compounds, especially 2, 4-D and 2-methyl-4-chlorophenoxyacetic acid(Alexander, 1969 ; Evans *et al.*, 1971), a finding which indicates rapid evolution and dissemination of these degradative properties in bacterial populations. Fisher *et al.*(1978) reported the involvement of plasmid -borne genes in the degradation of 2, 4-D by a strain of *Alcaligenes paradoxus*.

It is now known that many bacteria genera such as *Pseudomonas, Alcaligens, Flavobacterium*, etc. elaborate plasmids that encode a complete or partial degradative path-

way for a number of naturally occuring and synthetic compounds(Farell and Chakrabarty, 1979 ; Negoro *et al.*, 1980). Kamp and Chakrabarty(1979) reported on the occurrence of a transmissible plasmid(pAC21) in a strain of *Klebsiella pneumoniae*, isolated from the polychlorobiphenyl(PCB) -contaminated areas of the Hudson River, that encodes partial conversion of p -chlorobiphenyl(pCB) to p-chlorobenzoic acid (4Cba). Normally, members of enteric bacteria such as *K. pneumoniae* are incapable of utilizing hydrocarbons, and cannot express hydrocarbon-degradative genes when such genes are transferred as part of a degradative plasmid from *Pseudomonas*(Chakrabarty *et al.*, 1978). The evolutive pCB$^+$ *K. pneumoniae* can not only utilize pCB as a sole source of carbon, but can also produce mutant colonies that can functionally express *Pseudomonas* degradative plasmids such as TOL(Kamp and Chakrabarty, 1979).

In this report, we describe on the properties of nitrogen-fixing *Klebsiella pneumoniae* isolated from rhizosphere of rice, capable of utilizing chlorinated herbicides as a sole source of carbon and energy, and report that the degradation ability of chlorinated herbicides in *Klebsiella pneumoniae* is plasmid -borne.

MATERIALS AND METHODS

Bacterial strains

Details of the bacterial strains used for the work described in this paper are presented in Table 1.

Media and culture conditions

For the acetylene reduction assay, N-free medium(MacNeil *et al.*, 1978) was used. The contents are as follows: glucose, 6g; $MgSO_4$ $7H_2O$, 0.2g; Na_2HPO_4, 13.9g; KH_2PO_4, 1.7g; NaCl, 2g; $FeCl_3 \cdot 6H_2O$, 8mg; $Na_2MoO_4 \cdot 2H_2O$, 1mg; and distilled water to 1 liter. Luria-Bertoli broth(LB) was used for the test of resistance for antibiotic, herbicide and heavy metal ion. Minimal PAS medium(Chatterjee *et al.*, 1981) was used for the test of herbicide degradation. The contents are as follows: $K_2HPO_4 \cdot 3H_2O$, 2.96g; KH_2PO_4, 0.87g; NH_4Cl, 1.1g; $MgSO_4$, 0.097 g; $MnSO_4$ H_2O; 0.025g; $FeSO_4$ $7H_2O$, 0.005g; L- ascorbic acid, 0.005g; $CaCl_2H_2O$, 0.0015 g; distilled water, 1*l*; and adjusted to pH 7.0 Substrate(herbicide) was added 1*mg/ml*, but 100 $\mu g/ml$ for DCP, as sole sources of carbon and and energy, or with glucose(0.1%) as alternative carbon sources.

Response to antibiotics, heavy metal ion and herbicides

Resistance or sensitivity to antibiotic, herbi-

Table 1. *Bacterial strains.*

Strain	Genetic marker	Source or Reference
E. coli K060	Wild(C strain)	
K. pneumoniae M5a1	nif$^+$	Dixon & Postgate(1971)
K. pneumoniae KUNF1, KUNF2, KUNF3, KUNF5 KUNF6, KUNF7, KUNF8, KUNF9 KUNF10, KUNF11, KUNF12, KUNF13 KUNF14, KUNF15, KUNF16, KUNF17 KUNF18, KUNF19, KUNF25	nif$^+$	Hong & Lee(1985)
K. rhinoscleromatis KUNF26	nif$^+$	Hong & Lee(1985)
Enterobacter sp. KUNF4, KUNF20	nif$^+$	Hong & Lee(1985)
Unidentified sp. KUNF22, KUNF23, KUNF24	nif$^+$	Hong & Lee(1985)
K. oxytoca KUNF21	nif$^+$	Hong & Lee(1985)

cide or heavy metal ion was tested in LB broth by inoculating $50\mu l$ of overnight culture into $5ml$ of LB broth supplemented with the antibiotic, herbicide or heavy metal ion at the various range of concentrations, incubation for 24 hrs, with shaking at 150 rpm, and examining the turbidity indicating growth.

Acetylene reduction assay

Acetylene reduction assay was carried out in the test tube containing $5ml$ N-free medium. The strains were precultured in LB broth at 30℃ overnight with shaking. One-tenth ml of the preculture was inoculated into N-free medium in each tube containing 15% acetylene and 85% argon. The tubes were incubated at 30℃ for 48hrs. with shaking, and the ethylene produced was determined by gas chromatography.

Curing experiments

Cells were grown at 30℃ in LB broth supplemented with various concentration of mitomycin C for 48hrs. The cultures were then diluted and plated on LB agar. Colonies were then replica-plated to another LB agar plate and also to minimal PAS agar supplemented with appropriate substrates respectively to allow scoring and calculation of the proportion of substrate-negative segregants.

Detection of plasmid DNA

For detection of plasmid, gentle lysis method of the modified Eckhardt(1978) was carried out. After overnight culture, $0.5ml$ of the culture was washed with TE buffer(0.05M Tris, 0.02M EDTA·2Na, pH 8.0) incoporating 0.1% sarcosyl. 10^7 cells were resuspended in $20\mu l$ of 7% Ficoll, 20% sucrose, $1U/ml$ RNase and $1mg/ml$ lysozyme in TBE buffer(90mM Tris-base, 90 mM Boric acid, 2.5mM EDTA, pH 8.3). Cell suspension was put after mixing immediately into the well already filled with TBE buffer. It was carefully overlaid with about $50\mu l$ of 1% SDS, 5% sucrose, 5% bromophenol blue in TBE, and then agarose gel electrophoresis was carried out for 30mins at 5mA and 2hrs at 150V.

RESULTS AND DISCUSSION

Acetylene reduction activity

All the strains showed acetylene reduction activity under anaerobic condition(Table 2). *K. pneumoniae* KUNF9 had the highest nirogenase activity, but KUNF20, 21, 22, 23, 24, 25 and 26 had little nitrogenase activity. Originally, KUNF20-26 strains grew well on N-free agar medium when they were just isolated from the rice rhizosphere. The reason

Table 2. *Nitrogenase activity of nitrogen-fixing bacteria.*

Strain	Nitrogenase activity (n mole C_2H_4/hr/1 OD)
KUNF 1	50.22
2	50.74
3	80.76
4	112.88
5	35.89
6	46.37
7	66.11
8	131.72
9	154.95
10	65.26
11	108.05
12	38.89
13	35.89
14	20.31
15	51.26
16	69.63
17	87.23
18	82.26
19	50.64
20	7.48
21	4.55
22	1.50
23	0.73
24	0.17
25	0.15
26	0.34
M5a1	30.46
K 060	0

Table 3. *Resistance to antibiotics of the selected strains*

Antibiotic ($\mu g/ml$)*	Ap (1600)	Cm (25)	Gm (6.25)	Km (25)	Sm (6.25)	T_c (50)
KUNF 3	+	−	−	+	+	+
4	+	−	−	−	+	+
8	+	−	−	−	+	−
9	+	−	−	−	+	+
11	+	+	−	−	+	+
16	+	−	−	−	+	+
17	+	−	−	−	+	+
18	+	−	+	−	+	+

*Ap: ampicillin, Cm: chloramphenicol, Gm: gentamycin, Km: kanamycin, Sm: streptomycin, Tc: tetracycline

why they did show little nitrogenase activity was not clear.

For further study, we selected KUNF3, 4, 8, 9, 11, 16, 17 and 18 having high nitrogenase activity.

Response to antibiotics, heavy-metal ion and herbicides

All the strains showed various degrees of resistance to at least two and at most 3 or 4 of the 6 antibiotics tested(Table 3). Almost all of the strains were resistant to ampicillin, streptomycin and tetracycline, but were sensitive other antibiotics.

All the strains showed resistances to 2, 4-D (2, 4-dichlorophenoxy acetic acid), MCPA (2-methyl 1-4-chlorophenoxy acetic acid) and 3CB (3-chlorobenzoic acid), but not to DCP (2, 4-dichlorophenol)(Table 4). Due to toxicity of DCP compared with other herbicides tested all the strains did not grow at $400\,\mu g/ml$. When the concentration was lowered to $100\,\mu g/ml$, all the strains could be resistant.

All the strains tested were resistant to 100 μM of $HgCl_2$(Table 5).

Utilization of herbicides

In an attempt to verify the resistance to herbicides, several other chlorinated herbicides were tested for degradation. Patterns of chlorinated hydrocarbon utilization are shown in Table 6. All of the strains except KUNF4 could utilize the tested herbicide as sole source of carbon and energy. Normally, members of enteric bacteria such as *K.*

Table 4. *Resistances of the selected strains to herbicides*

Strain	2, 4-D	MCPA	3cB	DCP
KUNF 3	−	+	+	−
4	−	+	+	−
8	+	+	+	−
9	+	+	+	−
11	+	+	+	−
16	+	+	+	−
17	+	+	+	−
18	+	+	+	−

*The concentration of herbicides was 400 $\mu g/ml$ (in LB)

Table 5. *Resistance of the selected strains to mercuric chloride*

Strain	$HgCl_2$ (μM)	
	100	400
KUNF 3	+	−
4	+	−
8	+	−
9	+	−
11	+	−
16	+	−
17	+	−
18	+	−

Table 6. *Patterns of utilization of herbicides by the selected strains.*

Strain	2, 4-D	MCPA	3 cB	DCP
KUNF 3	+	+	+	+
KUNF 4	−	−	−	−
KUNF 8	+	+	+	+
KUNF 9	+	+	+	+
KUNF11	−	+	+	−
KUNF16	+	+	+	−
KUNF17	+	+	+	+
KUNF18	+	+	+	+

*The concentration of herbicides was 1 mg/ml, but 100 μg/ml for DCP in PAS medium.

pneumoniae are incapable utilizing hydrarbons, and cannot express hydrocarbon -degradative gens when such genes are transferred as part of a degradative plasmid from *Pseudomonas* (Chakrabarty *et al.*, 1978). But Kamp and Chakrabarty (1979) isolated p-chlorobiphenyl - degrading *K. pneumoniae* from *PCB*-contaminated areas of the Hudson River, and they found that the gene biodegradation exists on the transmissible plasmid pAC27. They have also reported that pAC21

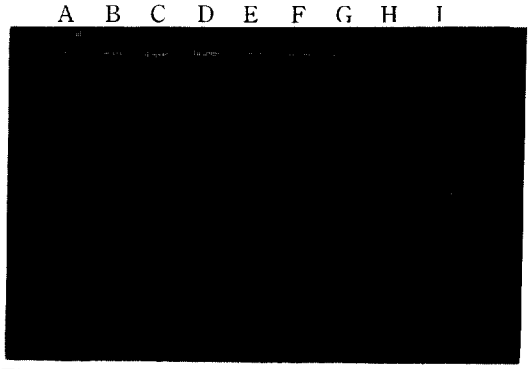

Fig. 1. *Plasmids from selected strains after separation by agarose gel electrophoresis.*
(A) KUNF3, (B) KUNF4, (C) KUNF8, (D) KUNF9 (E) KUNF11, (F) KUNF16, (G) KUNEF17, (H) KUNF18, (I) pAC27.

Table 7. *Curing or the selected Klebsiella strains by mitomycin C.*

Strain	Mitomycin C (μg/ml)	Number of colonies tested	Used marker for curing	Frequency of* curing (%)
Klebsiella pneumoniae			2, 4-D, 3cB	
KUNF 3	7.5	400	MCPA	0
KUNF 8	7.5	500	〃	0
KUNF 9	5.0	500	〃	0
KUNF11	2.5	500	〃	0
KUNF16	7.5	500	〃	11.0
KUNF17	7.5	400	〃	0
KUNF18	7.5	500	〃	0
Enterobacter sp.				
KUNF4	5.0	500	〃	0

*Curing frequency was calculated from the minimal PAS agar supplemented with 2, 4-D, 3CB or MCPA respectively.

is essential to allow *K. pneumoniae* to express functionally hydrocarbon degradative genes. Thus, it may be that our nitrogen fixing *K. pneumoniae* evolved as a bacteria having ability to degrade the chlorinated herbicides under herbicide-contaminated environment.

Detection of plasmid and curing of the degradative ability

Selected strains were tested for the presence of plasmids. All were shown to contain one plasmid by our extraction procedure. In Fig. 1, selected plasmids are shown after separtion by gel electrophoresis. The size of the plasmids was similar with pAC27 (110 kb). After these strains are treated with mitomycin C, cured strains are not able to grow on media containing herbicide as sole carbon sources. They therefore appear to have lost the herbicide degradative ability. This suggest that the degradative ability of herbicide may exist on their plasmid(Table.7).

적 요

벼의 근권으로부터 분리한 *Klebsilla pneumoniae* 가 2, 4-D, MCPA 및 3CB등 염소계 제초제들을 분해하여 단

일탄소원으로써 이용함을 관찰하고, 그들의 제초제 분해능이 플라스미드 유전자에 기인함을 전기영동에 의한 플라스미드의 분리 및 curing 실험을 통하여 확인하였다. 또한 이들 균주들의 질소고정능과 항생물질, 및 중금속이온에 대한 내성등 유전적 특징을 조사하였다.

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